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Role of hydrogen in the activation and regulation of hydrogen oxidation by the soluble hydrogenase from *Alcaligenes eutrophus* H16

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The activation kinetics of the H_2-oxidizing activity of the soluble hydrogenase from *Alcaligenes eutrophus* H16 were investigated. Activation with $Na_2S_2O_4$ plus 101 kPa H_2 resulted in a rapid increase in activity over 1 h and constant activity after 3 h incubation. Less-stable activations were achieved if enzyme was incubated with $Na_2S_2O_4$ under 1 kPa H_2 or 101 kPa N_2. The enzyme could also be partly activated either with NADH alone or with H_2 alone. The level of activity obtained with both 101 kPa H_2 and NADH present was greater than that obtained with either 101 kPa H_2 or NADH alone. Activation with H_2 plus NADH was virtually independent of NADH concentration but highly dependent on H_2 concentration. The effects of various concentrations of H_2 and constant concentration of NADH on the level of activation were the same whether H_2 oxidation was assayed by H_2-dependent Methylene Blue or NAD^+ reduction. Diaphorase activity did not require activation and was little affected by the treatments that activated H_2-oxidizing activity. The results suggest that H_2 plays an important role in regulating the level of H_2-oxidizing activity in this soluble hydrogenase.

INTRODUCTION

The soluble hydrogenase (H_2:NAD^+ oxidoreductase, EC 1.12.1.2) from the hydrogen bacterium *Alcaligenes eutrophus* H16 is a complex tetrameric enzyme (Schneider & Piechulla, 1986). Each tetramer contains Fe–S centres, Ni and FAD as prosthetic groups (Hornhardt *et al.*, 1986). The enzyme exhibits both NAD^+-dependent H_2 oxidation and NADH dehydrogenase (diaphorase) activity under appropriate conditions. H_2 oxidation can also be assayed independently of NAD^+/NADH binding by the use of artificial electron acceptors such as Methylene Blue (Schneider & Schlegel 1976).

The soluble hydrogenase from *A. eutrophus* H16, like many other aerobically purified hydrogenases, requires activation before H_2-oxidizing activity is observed. This soluble hydrogenase is remarkably stable towards O_2 when in the inactive, oxidized, state, but is rendered O_2-sensitive upon activation. Therefore the removal of O_2 as part of, or preceding, the activation is required (Schneider & Schlegel, 1976, 1981). The enzyme can be activated for H_2 oxidation (usually under anaerobic conditions) with H_2 alone (Egerer *et al.*, 1982), NADH plus H_2, NAD^+ plus H_2, $Na_2S_2O_4$, u.v. irradiation or glucose plus glucose oxidase (Schneider & Schlegel, 1976). The levels of activation achieved with these treatments have not been compared. A pivotal catalytic role for NADH in the activation of H_2-oxidizing activity has been suggested by Schneider & Schlegel (1976). When inactive enzyme is assayed with NAD^+ plus H_2, a lag phase is observed before NAD^+-dependent H_2 oxidation begins. This lag phase can be eliminated by including catalytic concentrations of NADH.

Once activated, this enzyme is reported 'unusually' unstable (Schneider & Schlegel, 1976). This instability accounts for up to a 19% loss of H_2-oxidizing activity after incubation for 24 h at 4 °C in the presence of 101 kPa H_2 plus 25 μM-NADH (Schlegel *et al.*, 1981). In the present paper we have compared the effects of some of the activators described above in terms of their effects on the stability of the H_2-oxidizing activity of the soluble hydrogenase from *A. eutrophus* H16. The results suggest that H_2 performs an important regulatory role in both the activation and stability of H_2-oxidizing activity.

MATERIALS AND METHODS

Purification of the soluble hydrogenase

The soluble hydrogenase was purified by using a modification of the method described by Schneider *et al.* (1983) in which the ion-exchange chromatography column was preceded by the Procion Red affinity column. Peak activity fractions eluted from the second column exhibited specific activities of 30–65 μmol of H_2 oxidized/min per mg of protein after activation for 2 h with 101 kPa H_2 plus 1 mM-$Na_2S_2O_4$ [spectrophotometric assay monitoring Methylene Blue reduction (85 μM) at pH 8.0 and 30 °C]. Peak fractions were then dialysed at 4 °C against 50 mM-potassium phosphate buffer, pH 7.0, and then stored under air at −20 °C. After prolonged storage (> 1 year), the enzyme was activated and reassayed as above and exhibited specific activities in the range 20–40 μmol of H_2 oxidized/min per mg of protein. SDS/polyacrylamide-gel-electrophoretic analysis of these samples revealed that the originally nearly homogeneous protein had undergone a limited degree of degradation during storage. Although the specific activities of these

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preparations are low relative to those described by Schneider *et al.* (1983), the experiments described in the present paper have made use of enzyme preparations of consistent activity so as to enable comparison between the various activation methods employed.

Incubation conditions during activation

Before activation the enzyme solution (1 mg of total protein/ml) was deoxygenated at room temperature by five cycles of evacuation and re-equilibration with O_2-free N_2. Unless otherwise stated, hydrogenase was then activated at room temperature in the inner chamber of a stoppered anaerobic double-chambered 13 ml serum-type incubation vial. To the outer chamber was added 0.5 ml of 0.1 M-$Na_2S_2O_4$ in 50 mM-Tris/HCl buffer, pH 8.0, to act as an O_2 scavenger. Additions to the enzyme solution were made from freshly prepared anaerobic stock solutions. Added volumes did not exceed 1% of the final volume. Mixtures of H_2 and N_2 in the incubation vials were generated by using a Matheson Dynablender gas mixer (Matheson Gas Products, Buffalo, NY, U.S.A.).

Determination of hydrogenase and diaphorase activities

Hydrogenase activity was determined by spectrophotometric assays monitoring either H_2-dependent Methylene Blue or NAD^+ reduction; 50 mM-Tris/HCl buffer, pH 8.0 (at 22 °C), was used as buffer in all activity assays. H_2-dependent Methylene Blue-reducing activity was determined at 30 °C in stoppered 6 ml cuvettes containing 3 ml of H_2-saturated buffer and Methylene Blue (85 μM). Assays were initiated by addition of enzyme. Methylene Blue reduction was monitored spectrophotometrically by using an experimentally derived molar absorption coefficient (ϵ) of 32.8 $mM^{-1} \cdot cm^{-1}$ at 600 nm. The H_2-dependent NAD^+ reducing activity was assayed as described by Schneider & Schlegel (1976). The diaphorase activity of the enzyme was monitored as NADH-linked ferricyanide reduction as described by Schneider & Schlegel (1976). In all assays the rate was determined from the initial rate of reaction (0–10 s). All absorbance changes were recorded with a Beckman DU-7 spectrophotometer.

Chemicals

All gases were of high purity (> 99.99%) and were obtained locally. Residual O_2 was stripped from these gases by passage through a heated Cu-based catalyst (R3-11; Chemical Dynamics Corp., South Plainsfield, NJ, U.S.A.). All other chemicals used were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Activation of H_2-oxidizing activity with $Na_2S_2O_4$

The time courses for the activation of H_2-dependent Methylene Blue-reducing activity of the soluble hydrogenase when incubated with $Na_2S_2O_4$ under various gas phases are shown in Fig. 1. Activity of initially inactive enzyme incubated with $Na_2S_2O_4$ (1 mM) under 101 kPa H_2 increased over the first 3 h to a stable level. The initial increase in activity of enzyme incubated with $Na_2S_2O_4$ under 1 kPa H_2 (balance N_2) was similar during the first 60 min, but then activity was slowly and continuously lost over the next 4.5 h. After 6 h the activity was 75% of the activity of enzyme activated under 101 kPa H_2.

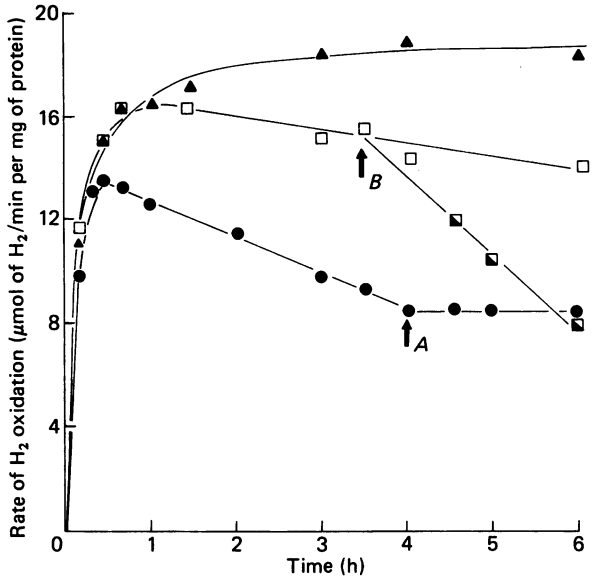

Fig. 1. Activation of H_2-oxidizing activity of *A. eutrophus* soluble hydrogenase with $Na_2S_2O_4$

Samples (200 μl) of anaerobic enzyme solution (1 mg of total protein/ml) were incubated in anaerobic incubation vials with 1 mM-$Na_2S_2O_4$ and a gas phase of 101 kPa H_2 (▲), 1 kPa H_2/100 kPa N_2 (□) or 101 kPa N_2 (●). At the indicated times 10 μl samples were removed and their H_2-oxidizing activities determined as H_2-dependent Methylene Blue reduction. At the arrow *A* 1 kPa H_2 was added. At the arrow *B* a duplicate vial was evacuated and refilled with O_2-free N_2 (◪).

Removal of the H_2 during this gradual loss of activity resulted in a more rapid loss of activity (Fig. 1). Enzyme activated with $Na_2S_2O_4$ in the presence of 101 kPa N_2 also lost activity at a similar rate after an initial activation phase. Addition of 1 kPa H_2 after 4 h stabilized the remaining activity but resulted in no regain of activity over the next 2 h. If 101 kPa H_2 was added, no reactivation of the enzyme could be detected within 1 h. However, up to 90% of the lost activity could be recovered after 12 h incubation. Additional experiments showed that the rate of activation and the final level of activity reached were independent of $Na_2S_2O_4$ concentrations from 0.01 to 2.5 mM (results not shown).

Activation of H_2-oxidizing activity with NADH and H_2

The time courses for the activation of H_2-dependent Methylene Blue-reducing activity of hydrogenase incubated with various concentrations of NADH with or without H_2 are shown in Fig. 2. For enzyme activation with NADH plus 101 kPa H_2, the rate of activation and final level of H_2-oxidizing activity were independent of NADH concentration, even when the NADH concentrations were approximately stoichiometric with enzyme. In the absence of H_2 (N_2 gas phase), activation with NADH resulted in an initial burst of activity, which declined to a lower, stable, activity after approx. 30 min. Activity was only 30% higher when enzyme was incubated with 50 μM-NADH rather than 0.5 μM-NADH. When enzyme was activated with H_2 (101 kPa) alone there was a lag phase of 30 min before the activity sharply increased from zero to a stable level. The final

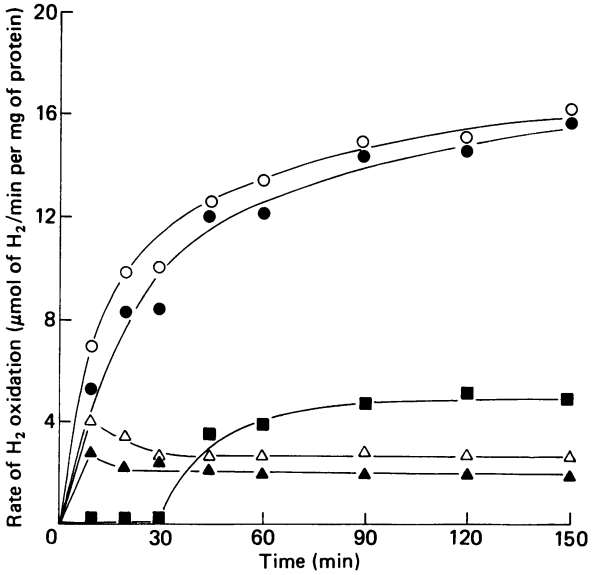

Fig. 2. Activation of H_2-oxidizing activity of *A. eutrophus* soluble hydrogenase with H_2 and NADH

The experimental conditions were as for Fig. 1 except that the incubation mixtures contained, in addition to enzyme, 0.5 μM-NADH plus 101 kPa H_2 (●), 50 μM-NADH plus 101 kPa H_2 (○), 0.5 μM-NADH plus 101 kPa N_2 (▲), 50 μM-NADH plus 101 kPa N_2 (△) or 101 kPa H_2 (no NADH) (■).

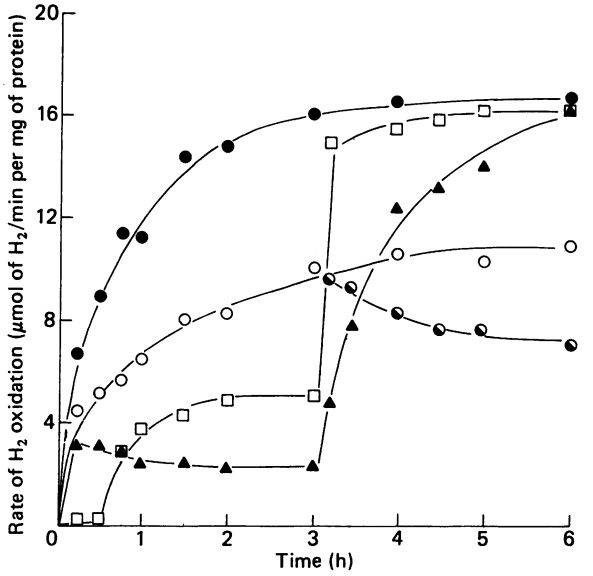

Fig. 3. Interconversion of activated states of *A. eutrophus* soluble hydrogenase

The experimental conditions were as for Fig. 1 except that the incubations contained, in addition to enzyme, 50 μM-NADH plus 101 kPa H_2 (●), 50 μM-NADH plus 1 kPa H_2/100 kPa N_2 (○), 50 μM-NADH plus 101 kPa N_2 (▲) or 101 kPa H_2 alone (no NADH) (□). After 3 h of incubation, the following changes were made: ▲, gas phase changed to 101 kPa H_2; □, addition of $Na_2S_2O_4$ (1 mM final concentration); ◐, removal of H_2 and replacement by 101 kPa N_2.

activity was approximately twice that of enzyme activated with NADH alone, but only approx. 30% of that exhibited by enzyme activated with NADH plus 101 kPa H_2.

The hydrogenase was activated at the same rate and to the same final activity with either NAD^+ or NADH when in the presence of H_2. However, in the strict absence of H_2 the hydrogenase was not activated by 50 μM-NAD^+ alone (results not shown). The rate of activation with H_2 alone (no added NADH) was dependent on the protein concentration of the enzyme solution; increased protein concentrations resulted in a faster activation. The H_2 concentration also affected the activation; lower concentrations gave lower rates of activation than did higher concentrations. However, neither the protein concentration nor the H_2 concentration affected the final specific activity of the enzyme in the absence of NAD^+/NADH (results not shown).

Interconversion of activated states

To determine if the activity state of hydrogenase produced under one set of conditions could be converted into another state by manipulation of incubation conditions, the experiment described in Fig. 3 was performed. Enzyme was activated with NADH plus various concentrations of H_2 and N_2 for 3 h. After this, the incubation conditions were altered and the effect on hydrogenase activity was determined. Enzyme activated with NADH plus 1 kPa H_2 reached a stable activity at 6 h that was about 70% of that obtained for the enzyme activated with NADH plus 101 kPa H_2. The removal of the H_2 from a duplicate incubation (1 kPa H_2) resulted in a non-linear loss of activity over the following 3 h. The activity did not tend towards zero but appeared to approach a stable lower level. Addition of $Na_2S_2O_4$ (1 mM final concentration) to hydrogenase initially activated with H_2 alone led to a rapid increase in activity that stabilized at the same level as that exhibited by hydrogenase originally activated with NADH plus 101 kPa H_2. For hydrogenase originally activated with NADH alone (N_2 gas phase), the low level of stable activity observed after 3 h was increased when the N_2 gas phase was replaced with H_2. The activity increased more slowly than with $Na_2S_2O_4$ but the final activity was again equivalent to that for enzyme originally activated with NADH plus 101 kPa H_2.

Effect of H_2 concentration on activation with NADH

Whereas activation with NADH plus H_2 appeared to be independent of NADH concentration, Fig. 3 suggested that the H_2 concentration could affect the final level of activity exhibited by this enzyme. To quantify this effect, hydrogenase activity was measured after incubation for 20 h in the presence of NADH plus H_2 pressures ranging from 0 to 101 kPa. Fig. 4(*a*) shows, like Figs. 2 and 3, that the hydrogenase could be activated in the presence of NADH alone (N_2 gas phase). As the H_2 concentration in the gas phase above the incubation was increased, the H_2-oxidizing activity also increased. Maximal activity was observed for enzyme activated in the presence of NADH plus 101 kPa H_2. NADH disappearance during such an incubation is less than 50% (final NADH concentration 28 μM). The maximal level of activity shown in Fig. 4(*a*) (NADH plus 101 kPa H_2) is somewhat less than that achieved during shorter incubations. This

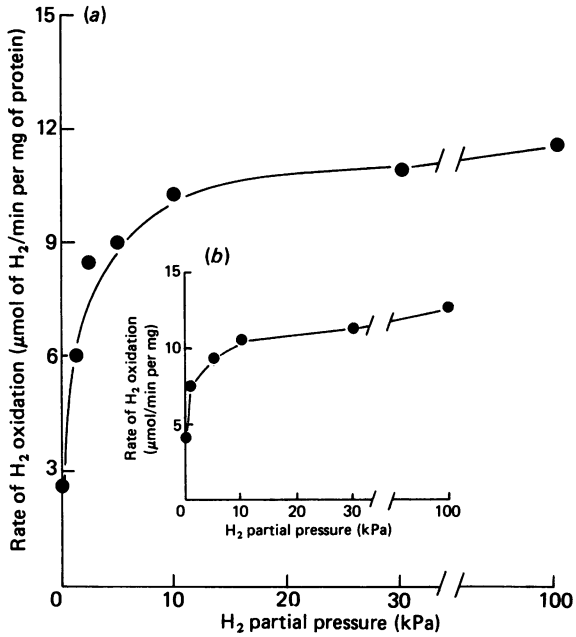

Fig. 4. Effect of H_2 concentration on activation of *A. eutrophus* soluble hydrogenase with NADH

Samples (200 μl) of anaerobic enzyme solution (1 mg of total protein/ml) were incubated for 20 h in anaerobic vials in the presence of 50 μM-NADH and the indicated pressures of H_2 (balance to 101 kPa was N_2). H_2-dependent Methylene Blue-reducing activity was then determined. (*b*) The enzyme sample activated for (*a*) in the presence of 101 kPa H_2 plus 50 μM-NADH was evacuated to remove the H_2 and then transferred to anaerobic vials with the indicated H_2 concentrations. After further incubation for 20 h, the H_2-oxidizing activity was determined.

suggests that prolonged incubation (> 6 h) under activating conditions can result in a partial loss of activity similar to that reported by Schlegel *et al.* (1981).

Fig. 4(*b*) shows the rates of H_2 oxidation exhibited when samples of hydrogenase activated with NADH plus 101 kPa H_2 for 20 h were evacuated (to remove the H_2), flushed with N_2 and then incubated for a further 20 h in vials containing 0–101 kPa H_2. The activity of these hydrogenase samples changed in response to the H_2 concentration in the second incubation. The activities exhibited for a given H_2 concentration in both Figs. 4(*a*) and 4(*b*) were similar, even at low H_2 concentrations.

The effects of activation of the H_2-oxidizing activity are apparently restricted to the portions of the hydrogenase that are involved in H_2 oxidation (Table 1). The differences in the rates of H_2-dependent Methylene Blue reduction exhibited by hydrogenase activated under various conditions used in Fig. 4 were also observed when H_2-oxidizing activity was assayed by monitoring NAD^+ reduction. No H_2-oxidizing activity was observed for hydrogenase incubated under strictly anaerobic conditions for 20 h (no NADH, N_2 gas phase). However, after a short lag phase (up to 1 min) this apparently inactive enzyme started to exhibit H_2-dependent NAD^+ reduction. This effect is presumably due to autocatalytic activation of the hydrogenase resulting from the generation of NADH in the assay cuvette. No such effect was observed in H_2-dependent Methylene Blue reduction

Table 1. Effect of activation on H_2-oxidizing and diaphorase activity

Four samples (200 μl) of anaerobic enzyme solution (1 mg of total protein/ml) were placed in the inner chamber of anaerobic incubation vials. The enzyme was then treated as indicated for 20 h at 4 °C. Samples of enzyme were then assayed for various activities as described in the Materials and methods section. The sample sizes were 20 μl for the Methylene Blue-reduction assay and 50 μl for both the NAD^+-reduction and diaphorase assays. The values in parentheses are from a separate experiment with enzyme of higher specific activity.

	Activity (μmol of substrate/min per mg of enzyme)		
Activation treatment	H_2-linked Methylene Blue reduction	H_2-linked NAD^+ reduction	Diaphorase activity
101 kPa N_2, no NADH	0 (0.2)	0.2	53.2
101 kPa N_2 + 50 μM-NADH	3.1 (5.4)	4.0	50.5
1 kPa H_2 + 50 μM-NADH	6.1 (16.8)	5.8	46.7
101 kPa H_2 + 50 μM-NADH	12.7 (37.4)	12.3	46.6

assays. The various activation states were also observed with enzyme of substantially higher specific activity (Table 1, values in parentheses).

In contrast with the effects of H_2 and NADH on the H_2-oxidizing activity of the hydrogenase, incubation of enzyme with NADH in the presence or in the absence of H_2 had no significant effect on the diaphorase activity of the enzyme (Table 1). The diaphorase activity, unlike the H_2-oxidizing activity, did not require activation and exhibited maximal activity before any treatment used to activate H_2-oxidizing activity.

DISCUSSION

In this study we have characterized the role of H_2 in the regulation of both the activity and the stability of H_2 oxidation catalysed by the soluble hydrogenase from *A. eutrophus* H16. When H_2-oxidizing activity was activated by $Na_2S_2O_4$ plus H_2 (Fig. 1), the initial level of activation (up to 90 min) was independent of the H_2 concentration (either 1 or 101 kPa). The removal of H_2 from the 1 kPa H_2 incubation led to a progressive loss of activity, suggesting that H_2 is important in maintaining the activity in $Na_2S_2O_4$-activated enzyme. This point was emphasized by the activation conducted in the presence of 101 kPa N_2. The lower maximal activity of this incubation, the stabilization against the further loss of the residual activity by the addition of low concentrations of H_2 (1 kPa) and a long-term re-activation in the presence of high H_2 concentrations (101 kPa) all point to a role for H_2 in both the activation process and the maintenance of activity when hydrogenase is activated with the strong reductant $Na_2S_2O_4$.

Activation of hydrogenase with combinations of the physiologically relevant reductants, NADH and H_2, is

more complex. The enzyme can apparently be activated to three kinetically distinct states, namely the two dissimilar low-activity states achieved by activation with NADH or H_2 alone (Fig. 2) and a high-activity state achieved by activation of the enzyme by the more than additive effects of both NADH and H_2 (Figs. 2 and 3).

The pivotal catalytic role of NADH in the activation of this enzyme was described in the Introduction. Our results (Fig. 2) confirm this role and suggest that activation with NADH plus H_2 (101 kPa) is independent of NADH concentration between 0.5 and 50 μM. Furthermore, NAD^+ could activate the enzyme in the presence of H_2 but not N_2. Our observation that the enzyme can be partially activated by NADH alone (Fig. 2) has not been reported before, although a modification of the e.p.r. signal by NADH alone was reported (Schneider *et al.*, 1979). The fact that the soluble hydrogenase slowly evolves H_2 in the presence of NADH (0.16 μmol/min per mg of protein in the presence of 2.4 mM-NADH; Schneider & Schlegel, 1976) is unlikely to account for this activation, given the low NADH concentrations used. Furthermore, the small increase in activation (30%) by a 100-fold increase in NADH concentration (0.5–50 μM) and the distinctive NADH-linked activation kinetics compared with those observed with H_2 alone suggest that H_2 evolution does not contribute to the activation of enzyme incubated with NADH alone. These results imply that the activations by NADH and by H_2 are through separate mechanisms. Activation of this hydrogenase with H_2 alone has been reported by Egerer *et al.* (1982) with enzyme of specific activity comparable with that used in the present study (20 μmol of H_2/min per mg of protein). These workers observed a lag phase during activation similar to that observed in the present study (Figs. 2 and 3). Activation with H_2 alone did not occur with immobilized hydrogenase. An intermolecular electron-transfer reaction between H_2-activated and unactivated hydrogenase molecules was proposed, which is in agreement with our observation that the enzyme concentration affects the rate of activation, but not the final level of activity achieved after incubation with H_2 alone.

Activation of this hydrogenase with both H_2 and NADH is kinetically distinct from the activations discussed above and produces a level of activity greater than the sum achieved with either activator alone (Figs. 2 and 3). This observation suggests a third, distinct, activation mechanism involving the concerted effects of both H_2 and NADH. Experiments to interconvert the enzyme activity between the proposed distinct activity states (Fig. 3) provided three important results. First, the levels of activation achieved with either $Na_2S_2O_4$ plus 101 kPa H_2 or NADH plus 101 kPa H_2 were the same. Secondly, the level of activation in the presence of NADH and H_2 was dependent on the H_2 concentration. Thirdly, removal of H_2 from enzyme activated with NADH plus H_2 resulted in a loss of activity, which did not, however, tend to zero.

With reference to the first point, Schneider *et al.* (1979) observed that the e.p.r. signal of the enzyme (as isolated) can be modified by NADH alone. Addition of H_2 to the NADH-reduced sample leads to a further modification of the signal that is almost identical with that observed for enzyme reduced directly with $Na_2S_2O_4$. Apparently, both forms of reduction lead to an equivalent fully reduced form of the enzyme. Our results indicate that activity measurements can be correlated with these biophysical observations; e.g. NADH-plus-H_2-treated hydrogenase oxidizes H_2 at the same rate as $Na_2S_2O_4$-plus-H_2-treated enzyme.

The effects of H_2 concentration on activation and stabilization were further characterized (Fig. 4). The removal of H_2 (but not NADH) from an NADH-plus-H_2-activated enzyme resulted in a decrease in enzyme activity to a stable level approximately equivalent to, but no less than, that achieved for hydrogenase activated with NADH alone. Apparently, the low level of activation achieved with NADH alone 'primes' the enzyme so that it can respond to the ambient concentration of H_2. The inter-relationship between NADH and the ambient H_2 concentration may be of physiological significance in regulating the activity of the soluble hydrogenase from *A. eutrophus* H16 *in vivo*. Under natural conditions the soluble hydrogenase is likely to be exposed to continuously varying concentrations of both NADH and H_2. The activation level of the enzyme is rather insensitive to NADH concentration (Fig. 2) but is acutely sensitive to H_2 concentration (in the presence of NADH), especially in the region of the reported K_m for H_2, 37 μM (Schneider & Schlegel, 1976) (Fig. 4). Furthermore, the response time of the hydrogenase to changes in H_2 concentration is sufficiently slow so as to enable the activity level to change in response to progressive long-term changes in the ambient H_2 concentration and not merely short-term fluctuations.

Although the kinetic analyses we have described in this paper have not made use of hydrogenase of the highest specific activity described in the literature (Schneider *et al.*, 1983), we have found that the activation effects obtained in Table 1 hold true for enzyme preparations of specific activity between 10 and 40 μmol/min per mg of protein (H_2-dependent Methylene Blue reduction) and for protein concentrations between 0.2 and 3.5 mg of total protein/ml (specific activity 20 μmol/min per mg of protein). Although it is possible that some of the effects that we have observed are due to the presence of contaminating proteins, it seems more plausible that our preparation contains inactive and/or degraded enzyme. Such protein is known to accumulate in whole cells and may not be completely removed by using affinity chromatography (Schneider *et al.*, 1983). The presence of catalytically incompetent enzyme would be compatible with both the low specific activity of our preparation and the observation that, where overlap exists, our results agree well with previously published kinetic analyses of this hydrogenase. One point of disagreement is our observation that this hydrogenase cannot be activated under simply anaerobic conditions (Table 1). Schneider & Schlegel (1976) reported activation with a glucose/glucose oxidase system to achieve anaerobicity. Perhaps the flavin-containing glucose oxidase acts as more than a simple O_2 scavenger and actively participates in the reduction of the hydrogenase, possibly through direct protein–protein interactions or loosely associated reduced flavin. It is of interest that Lissolo *et al.* (1984) were unable to activate the periplasmic hydrogenase from *Desulfovibrio gigas* after 12 h of flushing with O_2 and H_2-free N_2, whereas Berlier *et al.* (1982) were able to activate the same enzyme rapidly with a glucose/glucose oxidase system.

The apparent regulatory role of H_2 in the activation and maintenance of activity of *A. eutrophus* soluble

hydrogenase is also of significance to inhibitor studies of this enzyme. On the one hand, inclusion of H_2 during the incubation with inhibitor may mask the effects of inhibitors competitive with H_2. On the other hand, removal of H_2 leads to decreases in activity not related to the inhibitor. During this study we observed that both C_2H_2 and NO could act as inhibitors of the soluble hydrogenase. Details of the effects of NO are described in the following paper (Hyman & Arp, 1988). With C_2H_2, transfer of NADH-plus-H_2-activated enzyme to an H_2-free C_2H_2-filled vial led to complete loss of activity within 4 h, whereas enzyme transferred to an H_2-free N_2-filled vial lost only 60% of its activity over the same period. No inhibition by C_2H_2 was observed in the presence of H_2. The demonstration that activity of the soluble hydrogenase is highly dependent on the ambient H_2 concentration, and the methods used to activate the enzyme, requires that careful consideration must be paid to the interpretation of results obtained with this enzyme in the course of inhibitor and stability studies.

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